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Fertilization Between Closely Related Sea Urchins Is Blocked by Incompatibilities During Sperm-Egg Attachment and Early Stages of Fusion

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Abstract. Closely related sea urchin species in the genus *Echinometra* from Hawaii and Guam have strong species-specificity of fertilization. Crosses between the two species found in Hawaii, *E. mathaei* and *E. oblonga*, were compared in order to determine which steps of gamete interaction are responsible for fertilization barriers. The acrosome reaction, attachment of sperm to eggs, and fusion of sperm and egg membranes were measured in crosses between species and compared to within-species controls. In all crosses, eggs induced the acrosome reaction in 50–100% of sperm within 20 s. However, eggs bound about 3–5 times fewer heterospecific than conspecific sperm. In addition, electrical continuity between heterospecific gametes was achieved rarely under conditions that allowed conspecific gametes to achieve it readily. Only two sperm-egg fusion events were recorded in more than 80 min of heterospecific sperm interaction on 22 eggs. Accordingly, species-specific fertilization in these urchins results firstly from reduced attachment of the heterospecific sperm acrosomal process to the egg vitelline layer, and secondly from inability of attached heterospecific sperm to develop continuity with the egg plasma membrane. At both of these steps, incompatibilities are reciprocal. Thus a barrier to gene flow is mediated by molecular interactions during a specific part of the fertilization process, as the sperm acrosomal surface and the egg vitelline layer contact each other. Recognition molecules mediating these steps of fertilization may be capable of relatively rapid change, leading to species-specificity of fertilization.

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Introduction

The relationship between the evolution of reproductive isolation and species formation is complex and differs among taxonomic groups (Mayr, 1963; Donoghue, 1985; Carson, 1987; Kaneshiro and Boake, 1987; Templeton, 1989). Species are genetically distinct, yet they may sometimes be reproductively compatible. Although reproductive barriers do not always define species boundaries, populations of organisms that do become intrinsically reproductively isolated will usually evolve independently. Thus knowledge of the evolution of species-specificity at genetic loci involved directly in mate recognition could contribute to an understanding of biological diversification. Many studies have focused on aspects of species-specific premating recognition that partition gene pools. In most cases, recognition involves complex neurophysiological processes (see review by Boake, 1991), and the genetic basis of these processes remains obscure. In a few cases, recent work directly implicates specific genes involved in mate recognition behavior in maintaining reproductive isolation between species (*e.g.*, Wheeler *et al.*, 1991, reviewed by Coyne, 1992).

Species-specific fertilization is a type of mate recognition that is amenable to study because it involves direct interaction of recognition molecules on the surfaces of just two cell types, the sperm and the egg. Species-specific fertilization is particularly important in many different types of free-spawning organisms like sea urchins, which have relatively simple premating behavior prior to the

interaction of gametes. Incompatibilities between gametes from different species cause premating reproductive isolation and create barriers to gene flow (O'Rand, 1988; Minor *et al.*, 1989; Lessios and Cunningham, 1990; Vacquier *et al.*, 1990; Palumbi and Metz, 1991; Metz *et al.*, 1991). Although broad-scale allopatric models of speciation have been applied to marine organisms including sea urchins (Mayr, 1954), many examples are coming to light of closely related and cryptic marine species that have differentiated without obvious geographical barriers (reviewed by Knowlton, 1993). Rapidly evolving specificity in mate recognition processes, including gamete interaction, may be important in generating intrinsic reproductive isolation between populations and facilitating diversification of marine organisms (reviewed by Palumbi, 1992). An understanding of how species-specificity can arise in the fertilization process may contribute to an understanding of speciation.

We have focused a study of fertilization on closely related urchin species in the genus *Echinometra* that have developed strong reciprocal gamete incompatibilities. In these urchins, the specificity of fertilization contributes substantially to maintenance of reproductive isolation between species. Indo-Pacific species in the genus *Echinometra* are among the most closely related sea urchins known. These urchins are abundant in mixed populations, and they contain gonads with mature gametes over broadly overlapping seasons (Kelso, 1970; Uehara and Shingaki, 1985; S.R.P., unpublished data). They show only slight ecological or morphological differentiation (Kelso, 1970; Uehara and Shingaki, 1985) and are similar enough to have been previously classified as different morphs of a single species (Mortensen, 1943). Measurements of genetic relationships indicate that the species are distinct from one another and probably differentiated from a common ancestor during the Pleistocene (Palumbi and Metz, 1991). Within that time, species-specificity of fertilization has arisen, making many of the possible crosses between these urchins incompatible (Kelso, 1970; Branham, 1972; Uehara *et al.*, 1990; Palumbi and Metz, 1991). In previous studies of gamete compatibility between Hawaiian *Echinometra* species, we found that only a small percentage of the eggs in reciprocal test crosses between these species typically become fertilized (Palumbi and Metz, 1991). Here we have extended cross-fertilization measurements to include a third species, referred to as 'type A' (Uehara and Shingaki, 1985), from Guam. Like the two species found in Hawaii (*E. mathaei* and *E. oblonga*), the two species found in Guam (*E. mathaei* and *Echinometra* type 'A') have strong reciprocal barriers to fertilization.

Fertilization depends on successful completion of a series of steps involving interactions between gametes; incompatibilities at these steps could prevent fertilization and provide the basis of species-specific fertilization (Lillie, 1919). These steps involve recognition and binding of surface molecules on sperm and eggs (for review see Metz and Monroy, 1985; Schatten and Schatten, 1989; Foltz and Lennarz, 1993). After conspecific sperm approach the sea urchin egg, four major steps of gamete interaction occur: (1) components of the egg jelly induce the sperm acrosome reaction (Trimmer and Vacquier, 1986); (2) sperm and egg attach as a result of interaction of the sperm protein bindin and its egg surface receptor (Minor *et al.*, 1989; Foltz and Lennarz, 1993); (3) molecular interactions of the attached acrosomal and vitelline surfaces bring gamete plasma membranes together; and (4) the membranes fuse, creating continuity between the gametes (Whitaker *et al.*, 1989; Chambers, 1989; Foltz and Lennarz, 1993).

Specificity of fertilization in crosses between several genera of urchins has been shown to result mainly from "primary gamete binding," the attachment of the sperm acrosomal process to the egg vitelline layer, visualized by electron microscopy (Summers and Hylander, 1975). We used electron microscopy to quantify the acrosome reaction and sperm-egg attachment in crosses of Hawaiian *Echinometra* species. In addition, we examined the species-specificity of events that occur after sperm-egg attachment and lead to gamete fusion. Fusion of the sea urchin sperm and egg plasma membranes results in a rapid depolarization of the egg membrane (reviewed in Jaffe, 1986). When eggs are held at their resting transmembrane potential to prevent normal depolarization, temporary continuity between the sperm and egg creates transient currents across the egg membrane (Chambers, 1989; Whitaker *et al.*, 1989; Chambers and McCulloh, 1990). To assess the capability of sperm and egg plasma membranes to fuse, we measured the incidence of transmembrane currents in voltage-clamped eggs.

The step-by-step examination of gamete interactions between Hawaiian *E. mathaei* and *E. oblonga* shows that reciprocal incompatibilities occur at the attachment step and in the interactions of attached gametes leading to membrane fusion. Comparison of these results with those reported from other crosses both within and between urchin genera suggests that when gamete incompatibility arises between species, these steps of fertilization are most likely to be involved.

Materials and Methods

Urchins and gametes

Echinometra mathaei and *E. oblonga* (*sensu* Edmondson, 1946), were collected from mixed populations at Kapapa Island in Kaneohe Bay, Oahu, Hawaii, and maintained in seawater tables at the Kewalo Marine Laboratory. Urchins of *Echinometra* species type 'A' (*sensu* Uehara and Shingaki, 1985) were shipped by air

from Guam. Shedding of gametes was induced by injection of 0.5 *M* KCl into the coelomic cavity. Eggs were collected by inverting female urchins in dishes of seawater. Undiluted sperm was collected at the gonopores. Filtered (0.45 μm) seawater was used for washing and diluting gametes.

Measurement of cross-fertilization

Gametes were obtained from two males and two females of each of three species of *Echinometra* (*E. mathaei, E. oblonga,* and *Echinometra* type 'A'). The 36 possible crosses between these individuals were made as follows. Sperm concentrations were normalized by dilution with seawater to give an optical density reading of 0.5 in a spectrophotometer set at 340 nm. Eggs were washed twice by gentle centrifugation through seawater. A 20-μl volume of settled eggs was mixed with 200 μl of sperm suspension in a 1.5-ml microcentrifuge tube. Counts showed that these gamete mixtures contained about 300 sperm per egg (sperm were counted with the aid of a hemacytometer). Two replicate tubes were prepared for each cross. All crosses were performed with highly motile sperm within 2 h of induced spawning. After 10 min of gamete interaction, 50 μl of 5% glutaraldehyde in seawater was added to fix gametes in one tube; the other tube was fixed after 3 h of gamete interaction. Presence or absence of the fertilization envelope in the 10-min samples and presence or absence of cell division in the 3-h samples was recorded for all eggs in a haphazard succession of fields of view at 100× magnification under a light microscope, until at least 200 eggs had been examined for each sample.

Test crosses between *Echinometra* type 'A' and *E. mathaei* were also performed on sympatric urchins in Guam. In these crosses, percent fertilization was measured under conditions similar to those described above, for four to five different pairs of urchins in each direction.

Electron microscopy of Hawaiian Echinometra *Crosses*

For electron microscopy, each gamete mixture used for the crosses was composed of an approximately equal mixture of gametes from four individual urchins. Accordingly, in each cross the gamete interaction levels of 16 individual crosses were measured. This method will sample the cumulative results of all crosses between pooled individuals. Thus, if certain individual urchin pairs have high cross-fertilizability, estimates of the amounts of acrosome reaction and gamete attachment might be elevated compared to those from the average cross between one male and one female. Accordingly, our measurements can be considered to overestimate rather than underestimate the successful completion of each step of fertilization. This approach will be less likely to underestimate the potential for gene flow between species at the population level.

Eggs were washed twice by gentle centrifugation through seawater, and 200-μl aliquots of the egg pellet from four females were mixed to make a pooled sample of eggs for each species. Likewise, a pooled sperm sample was made for each species by mixing 10 μl of undiluted sperm from each of four individuals with 10 ml of seawater. Sperm concentrations were normalized by adding seawater to give an optical density reading of 1.0 at 340 nm. Each of the four possible crosses between the two pooled sperm samples and the two pooled egg samples was made as follows. A 3-ml volume of sperm suspension was added to 0.25 ml of packed eggs and inverted repeatedly to mix. Counts showed that these gamete mixtures contained about 1000 sperm per egg (sperm were counted with the aid of a hemacytometer). Sperm appeared highly motile when all crosses were made. The two interspecific crosses were made before the two intraspecific crosses. After 20 s of gamete interaction, the sample was fixed for 1 h at room temperature by mixing thoroughly with an equal volume of freshly prepared 5% glutaraldehyde in seawater. Glutaraldehyde fixes the jelly coat surrounding the egg and the sperm embedded in it, as well as the sperm bound to the egg surface. Unused pooled eggs were fixed in 1% glutaraldehyde in seawater as negative controls. No fertilization envelopes were seen in these control samples. The samples were postfixed with 1% OsO_4, block-stained with uranyl acetate, and processed for transmission electron microscopy.

Thin sections of gametes from the crosses were examined by electron microscopy at 10,000× magnification. Each sample was searched for sperm in which the tip appeared in longitudinal section near the egg surface. For convenience, a sperm was considered to be near the egg surface if the tip was within 5 μm of the egg surface. Sperm that have approached the egg surface this closely have presumably had an opportunity to interact with the egg jelly and undergo the acrosome reaction. Any sperm within 5 μm of the egg surface was counted if it had either a clearly visible acrosomal vesicle or an acrosomal process. Any sperm connected at the acrosomal process to the egg surface by densely staining material was considered to be attached. Each sperm tip found was scored as unreacted, acrosome reacted but unattached, or attached. For each sample cross, at least 35 sperm tips were scored.

This experiment was repeated as described above, using different individual urchins as the source of gametes. In the second experiment, the electron microscopy samples were fixed after 40 s of gamete interaction instead of 20 s. Because the experiment was repeated, a total of 32 urchin pairs were surveyed for each of the four possible crosses between the two species.

Although other methods for measurement of gamete interaction have been described (*e.g.*, Vacquier and Payne, 1973), examination of gamete interaction in the electron microscope allows the relative amounts of sperm undergoing different steps of fertilization to be visualized and measured within the same samples. For each egg sample, a *G*-test of independence (Sokal and Rohlf, 1981, pp. 735–738) was used to test the null hypothesis that the number of sperm completing each of the two steps (acrosome reaction and attachment) is independent of whether the sperm are conspecific or heterospecific (Figure 2).

Electrophysiological recordings of Hawaiian Echinometra *crosses*

Electrophysiological measurements were performed as previously described (Kane, 1990), with the following modifications. Microelectrodes filled with 0.5 *M* K_2SO_4, 20 m*M* NaDl, and 0.5 m*M* potassium citrate (Lynn and Chambers, 1984) had resistances of 20–30 mΩ. The bath electrode was Ag-AgCl with a seawater agar bridge. Electrical measurements and voltage clamping utilized an Axoclamp-2A amplifier with membrane potential and current displayed on a digital oscilloscope. The input of the sample-and-hold amplifier (monitor output) was continuously observed on another oscilloscope while the gain and phase of the clamp was adjusted. Eggs were impaled by means of a short (20 ms) oscillation induced by an increase in the negative capacitance. Only impaled eggs requiring less than 0.10 nA of current to maintain a transmembrane potential of −80 mV were used. Single-electrode voltage clamping (Wilson and Goldner, 1975; Finkel and Redman, 1984) was done at a switching frequency of 2 kHz and filtered at 200 Hz.

Undiluted "dry" sperm were stored on ice and diluted in seawater 2–3 min before use. Eggs were dejellied by vortexing them gently in 0.55 *M* NaCl, 0.01 *M* KCl, and 5 m*M* Tris pH 8.3. Dejellied eggs were placed in plastic petri dishes pretreated with 0.001% protamine sulfate to allow adhesion (Steinhardt *et al.*, 1971). As a positive control for fertilization, two to four dejellied eggs from the other *Echinometra* species were added to the dish near the egg to be impaled. The egg was then impaled and voltage clamped near the resting potential at −75 mV.

First, the heterospecific sperm were added to the impaled egg. A 2-μl volume of dry sperm from a heterospecific male was diluted into 40 ml of seawater and vigorously pipetted to mix. In some cases, 1 μl of dry sperm from each of four heterospecific males was diluted into 40 ml of seawater. In separate experiments, sperm concentration was increased fourfold: 8 μl of dry sperm was diluted into 20 ml of seawater. Transmembrane potential and current were recorded while 10-μl aliquots of sperm were added near the impaled egg. The same volume of sperm was added at intervals of about 1 min for 3–5 min. The impaled egg was observed under the microscope to verify that heterospecific sperm had arrived at the egg surface and that heterospecific gamete interaction occurred for several minutes. The time at which the positive control eggs (from the same species as the sperm) had elevated fertilization envelopes was noted. After an additional 1–3 min—to ensure that the impaled egg had been exposed to heterospecific sperm for long enough to undergo an electrical response—conspecific sperm were added to the impaled egg. A 2-μl volume of dry sperm from a conspecific male was diluted into 40 ml of seawater and added to the impaled egg in 10-μl aliquots about every 1–2 min for 2–5 min. The amount of conspecific sperm delivered to the dish never exceeded the amount of heterospecific sperm.

For each of the two *Echinometra* species, electrophysiological data were recorded for 11 eggs (one to three eggs from six to seven female urchins). Some of these eggs were treated with sperm from one heterospecific male at a time; the other eggs were treated with mixtures of sperm from four heterospecific males. Overall, we surveyed heterospecific crosses between 18 pairs of urchins for *E. mathaei* eggs, and 16 pairs for *E. oblonga* eggs.

Results

Cross-fertilizability of three species of Echinometra

Of the six possible crosses between *E. mathaei* and *E. oblonga* from Hawaii and *Echinometra* type 'A' (Uehara and Shingaki, 1985) from Guam, all but one were incompatible (Figure 1). Measurements of percent fertilization envelopes after 10 min were similar to measurements of percent dividing zygotes after 3 h. The mean of the 12 within-species crosses was 98% eggs with fertilization envelopes. Under these conditions, the mean fertilizability between *E. mathaei* and *E. oblonga* was 3% and the mean fertilizability between *E. mathaei* from Hawaii and *Echinometra* type 'A' from Guam was 1%. Low cross-fertilizability was also found in a separate set of crosses between sympatric *Echinometra* type 'A' and *E. mathaei* individuals from Guam. For these experiments, the mean and standard deviation of four to five crosses in both directions was 5 ± 6.5% (range <1%–20%). Accordingly, the sympatric species pairs found on Hawaii and on Guam are both reciprocally reproductively isolated at fertilization.

Substantial fertilization occurred in one of the crosses between allopatric populations of urchins. Sperm of *Echinometra* type 'A' from Guam were capable of fertilizing eggs of *E. oblonga* from Hawaii (Fig. 1). In this direction, fertilizability of eggs from different individual urchins was variable; cross-fertilization was 14–27% of eggs from one of the two *E. oblonga* females we tested and 95–97% in the other. This preliminary evidence of vari-

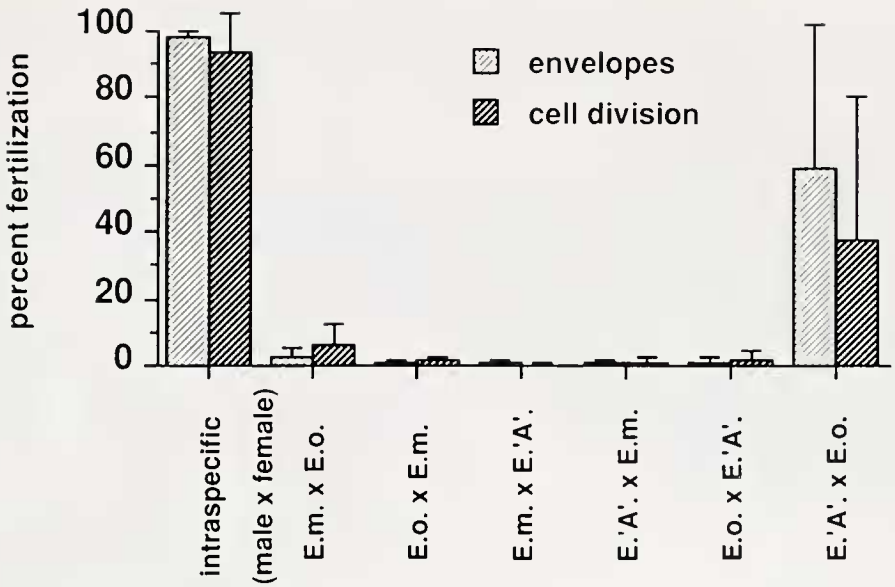

Figure 1. Cross-fertilization between *Echinometra mathaei* and *E. oblonga* from Hawaii and *Echinometra* species 'A' (Uehara and Shingaki, 1985) from Guam. All possible crosses between two males and two females of each species were made in duplicate with standardized gamete concentrations. For each heterospecific cross, the bars indicate the mean and standard error of four different crosses. Results of all 12 of the within-species crosses are shown together by the bars marked intraspecific. For each cross, the male is indicated first. E. m.: *E. mathaei;* E. o.: *E. oblonga;* E. 'A'.:*Echinometra* species 'A'.

ation in cross-fertilizability warrants further investigation. Although cross-fertilization between the two Hawaiian *Echinometra* species typically remains low even at high sperm concentrations (Palumbi and Metz, 1991), cross-fertilization between these allopatric urchins may depend more directly on sperm concentration.

Our results agree with results of a set of crosses made by Uehara *et al.* (1990) between *Echinometra* species in Okinawa. The Okinawan urchin phenotypically similar to Hawaiian *E. oblonga* is referred to as *Echinometra* type 'D'; comparisons of morphology and cross-fertilization are under way for these allopatric urchins (T. Uehara, pers. comm.). *E. oblonga* from Hawaii and *Echinometra* type 'D' from Okinawa showed low cross-fertilization with *E. mathaei* (Fig. 1, Uehara *et al.,* 1990), although more *Echinometra* type 'D' eggs than *E. oblonga* eggs may be fertilized by *E. mathaei* sperm. Substantial percentages of eggs from *E. oblonga* or *Echinometra* type 'D' were fertilized by sperm from *Echinometra* type 'A', whereas in both cases the reciprocal crosses were strongly blocked (Fig. 1, Uehara *et al.,* 1990). Finally, *E. mathaei* and *Echinometra* type 'A' were reciprocally incompatible in Okinawa (Uehara *et al.,* 1990), just as in our results for Guam and between Hawaii and Guam. Thus, in general, the patterns of fertilization between species appear to be similar both from crosses of sympatric urchins from different locations and for crosses between sympatric and allopatric populations of urchins.

Gamete interaction of Hawaiian Echinometra *species*

In all crosses between *Echinometra* species, sperm rapidly became embedded in the egg jelly. Electron microscopy revealed that in both heterospecific and conspecific crosses, sperm had arrived to within 5 μm of the egg vitelline layer within 20 s. Qualitatively, no differences were observed in the abundance of sperm at the egg surface in the different crosses.

More than 95% of sperm were acrosome-reacted within 20 s in three of the four possible crosses between the two Hawaiian *Echinometra* species (Fig. 2). A significantly reduced proportion of *E. mathaei* sperm approaching *E. oblonga* eggs underwent the acrosome reaction ($p < 0.005$, G-test of independence), indicating that in one direction there is specificity of the acrosome reaction. In this cross, the number of sperm acrosome-reacted at 40 s (72%) was higher than the number of sperm acrosome-reacted at 20 s (57%), suggesting a relatively slow rate of induction of the acrosome reaction compared to the other crosses.

In agreement with previous electron microscopy studies of sea urchin sperm, the acrosomal process of *Echinometra* sperm appeared to be coated with a densely staining granular substance, presumably including the protein bindin (Moy and Vacquier, 1979, Nishioka *et al.,* 1990). Sperm that had attached to the egg were connected to the vitelline layer by a continuous layer of this densely staining material (Fig. 3). Sperm in the process of fusing with eggs

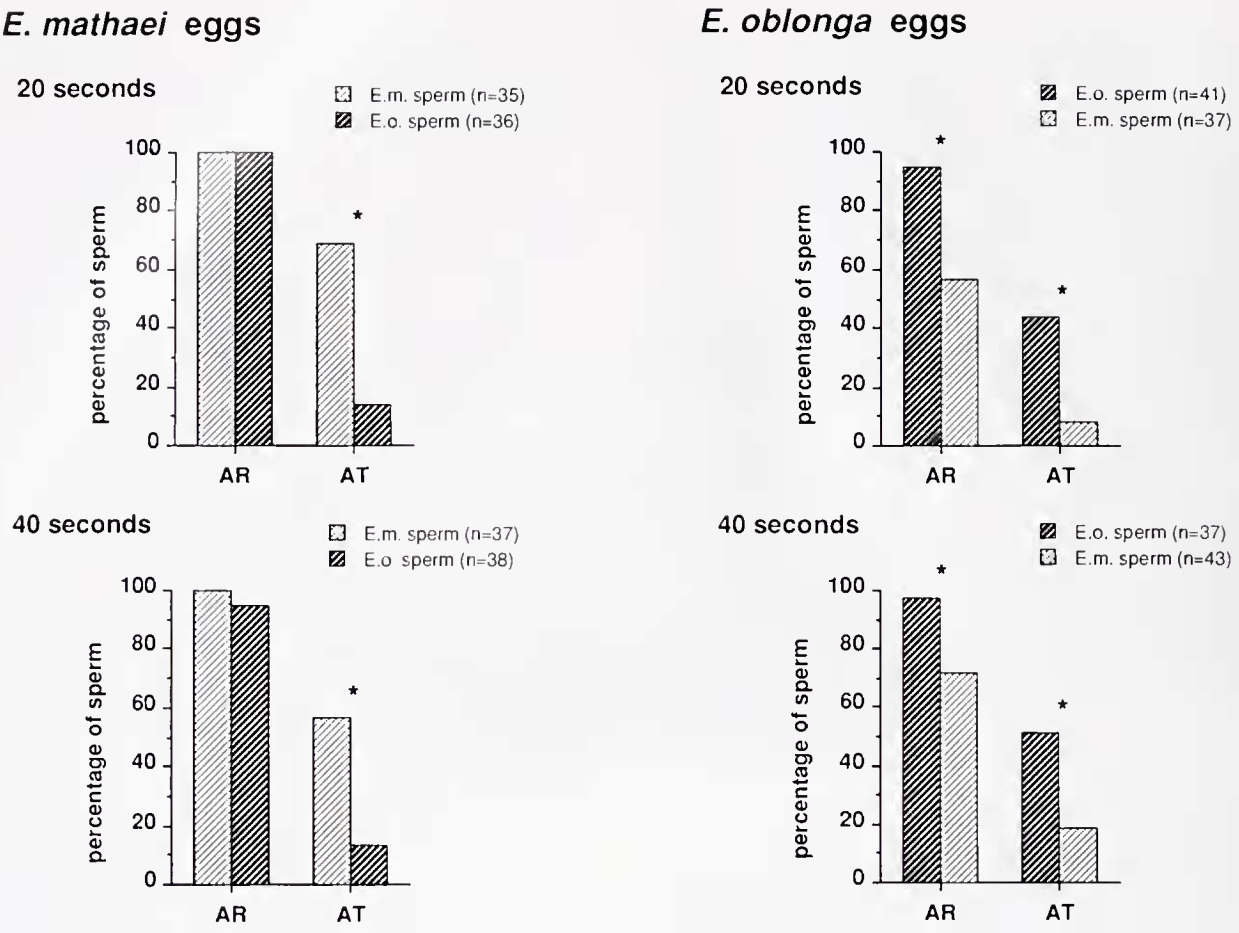

Figure 2. Measurements of gamete interaction in crosses between two *Echinometra* species. For eggs of each species, the percentages of conspecific and heterospecific sperm that underwent the acrosome reaction and became attached are shown for two experiments in which gamete interaction was allowed to occur for 20 s and 40 s, respectively. Different urchins were used in the two experiments. For each cross, the number of sperm, *n*, that were found in longitudinal section at the tip, within 5 μm of the egg surface, is indicated. AR: percentage of the *n* sperm that had undergone the acrosome reaction; AT: percentage of the *n* sperm that were attached to the egg vitelline layer by densely staining material; E. m.: *E. mathaei*; E. o.: *E. oblonga*.

For each egg sample, a *G*-test of independence (Sokal and Rohlf, 1981) was used to test the null hypothesis that the number of sperm completing each of the two steps (acrosome reaction and attachment) is independent of whether the sperm are conspecific or heterospecific. In comparisons marked with asterisks, the null hypothesis can be rejected. *:$p < 0.005$.

were observed in a few cases in the intraspecific crosses, but were not observed in any of the interspecific crosses.

In all cases, conspecific sperm demonstrated a significantly greater ability to attach to eggs than did heterospecific sperm ($p < 0.005$, *G*-test of independence). *E. mathaei* eggs bound 4.3–4.9 times more conspecific than heterospecific sperm; *E. oblonga* eggs bound 2.8–5.4 times more conspecific sperm than heterospecific sperm (Fig. 2). In each case, gamete interaction for the longer period resulted in relatively more heterospecific sperm becoming attached, suggesting that heterospecific sperm may attach at a slower rate than conspecific sperm.

Transient electrical continuity

After addition of heterospecific sperm to voltage-clamped eggs, about 3–10 sperm appeared to be in contact with each egg in the focal plane around the egg circumference. (Approximately 15–20 were seen when the sperm suspension added was fourfold more concentrated.) The total number of sperm contacting the egg was presumably several times higher. These heterospecific sperm appeared to be adhering to the surface of the dejellied egg; beating of flagella caused them to rotate around a contact point at their tip. In all cases, the positive control eggs (dejellied eggs from the same species as the sperm) had elevated

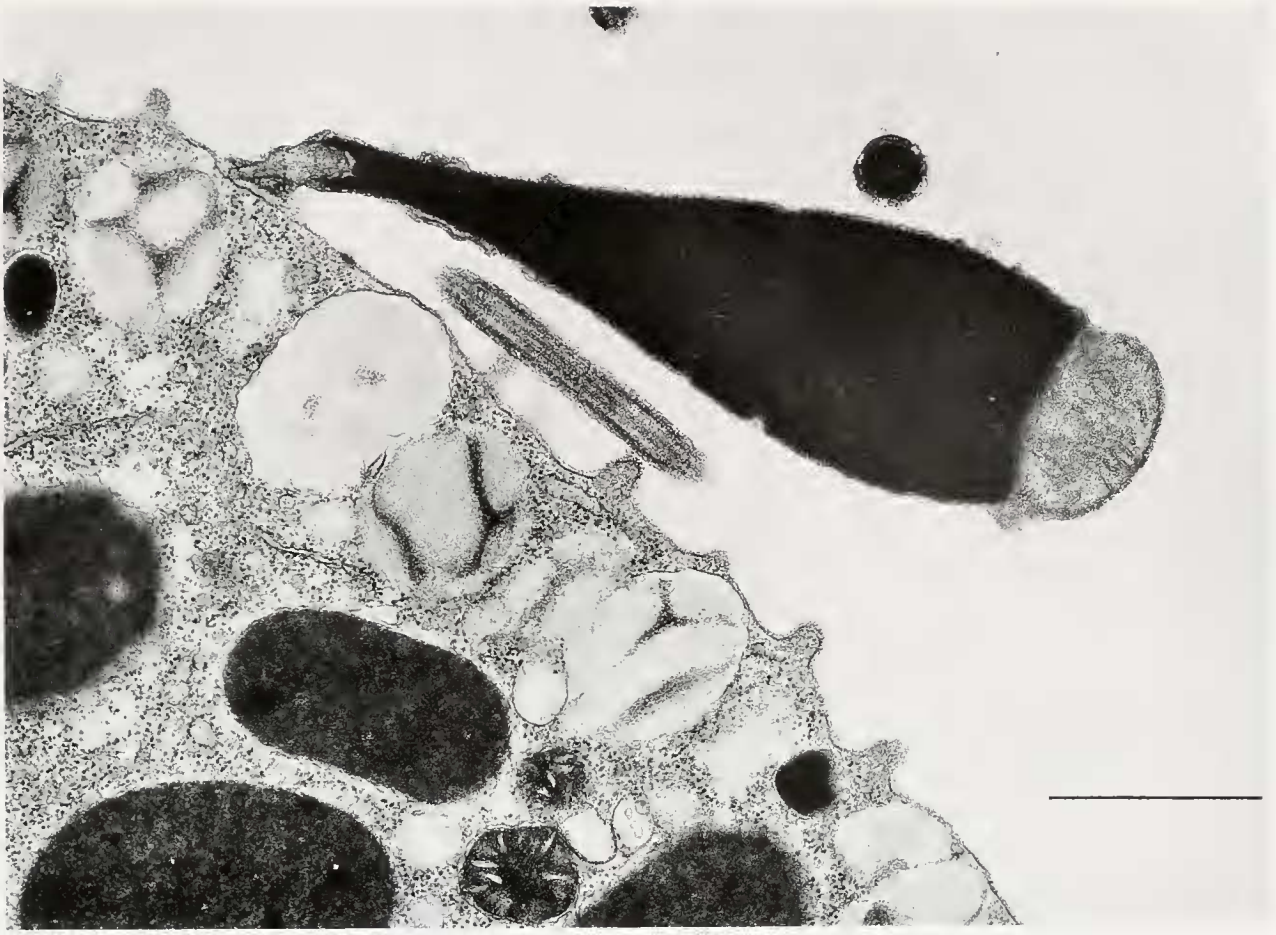

Figure 3. Electron micrograph of *Echinometra oblonga* sperm on *E. mathaei* egg fixed after 40 s of gamete interaction. Some heterospecific sperm-egg attachment occurs, but only rarely leads to gamete fusion. The acrosome is attached to the egg vitelline layer by densely staining acrosomal material, presumably bindin. (Bar = 1 μm.)

fertilization envelopes within 2–3 min, demonstrating that the heterospecific sperm were capable of fertilization.

Recordings totaling more than 80 min of heterospecific gamete interaction were taken from 22 eggs prior to addition of conspecific sperm; heterospecific sperm were allowed to interact for 2–6 min with each voltage-clamped egg. In spite of this opportunity for attachment and fusion, only two heterospecific transient current events occurred, both when *E. oblonga* eggs were exposed to concentrated *E. mathaei* sperm (Table I). For 20 out of 22 eggs, no electrical transients were observed for heterospecific sperm.

At an average of 30 s (range: 10–70 s) after addition of conspecific sperm near to voltage-clamped eggs, transient currents of 0.25–0.75 nA began to occur (Table I and Fig. 4). Under the conditions used for these experiments, the average number of transient current events per minute was similar in the two species: 1.9 for conspecific sperm on *E. mathaei* eggs and 1.4 for conspecific sperm on *E. oblonga* eggs. Occasionally, fusion with more than one conspecific sperm simultaneously was indicated by larger currents (Chambers and McCulloh, 1990). In most cases, the impaled eggs were then capable of undergoing a normal fertilization depolarization and elevation of the fertilization envelope when the voltage clamp was removed. The two heterospecific fusion events we recorded produced currents similar in magnitude and duration to those produced by conspecific fusion events.

Comparison of the two heterospecific crosses between Hawaiian *Echinometra* species shows that *E. oblonga* eggs appear to accept slightly more heterospecific fertilization (Fig. 1), heterospecific sperm attachment (Fig. 2), and heterospecific fusion (Table I), than *E. mathaei* eggs. However, the overall pattern in these urchins is strong reciprocal species-specificity of fertilization caused by

Table I

Incidence of transient currents in voltage-clamped eggs exposed first to heterospecific sperm and then to conspecific sperm

			Heterospecific sperm		Conspecific sperm		
Trial #	Female[1]	# males[2]	Time (s)[3]	Transients[4]	Time (s)[5]	Transients[6]	Fertilization[7]
E. mathaei eggs							
1	a	4	200	0	170	3	+
2	a	4	184	0	125	6	+
3	b	4	184	0	155	3	+
4	b	4	205	0	105	4	+
5	c	1	220	0	80	3	+
6	c	1	140	0	165	5	+
7	d	4	200	0	160	6	+
8	d	4	180	0	105	4	+
9	e	1 conc.	240	0	400	11	
10	f	4 conc.	295	0	145	7	
11	f	4 conc.	160	0	115	4	+
E. oblonga eggs							
1	a	1	225	0	145	4	+
2	b	1	275	0	130	3	+
3	b	1	185	0	95	2	+
4	b	1	180	0	130	4	+
5	c	4	275	0	95	3	+
6	c	4	195	0	135	3	+
7	d	4	200	0	85	3	+
8	d	4	225	0	90	3	+
9	e	1 conc.	380	0	300	5	
10	f	1 conc.	305	1	210	3	+
11	g	4 conc.	275	1	125	2	

[1] Each female from which eggs were obtained is designated by a different letter.
[2] Number of heterospecific males from which sperm was obtained; *conc.*: fourfold more concentrated sperm.
[3] Time in seconds of heterospecific gamete interaction before addition of conspecific sperm.
[4] Number of transient currents occurring prior to addition of conspecific sperm.
[5] Time in seconds between addition of conspecific sperm and removal of voltage clamp.
[6] Number of transients occurring after addition of conspecific sperm.
[7] After removal of the voltage clamp, normal fertilization depolarization and elevation of the fertilization envelope is indicated by a '+'.

strong reciprocal species-specificity of interactions as gametes attach and begin to fuse.

Discussion

Species-specificity of Echinometra *fertilization*

Of the many studies on sea urchin fertilization, few have examined species-specific fertilization step by step in crosses between congeners. Crosses of *Strongylocentrotus purpuratus* and *S. franciscanus* showed species-specificity of the sperm-egg attachment step but no specificity prior to attachment (reviewed in Minor *et al.*, 1989, 1991). Indo-Pacific *Echinometra* species are about 4–5 times more closely related than these *Strongylocentrotus* congeners (Palumbi and Metz, 1991) and provide a view of species-specific fertilization at a time much nearer the time of species formation. Barriers to fertilization have evolved in all but one of the six possible crosses between the three species of *Echinometra* found on Hawaii and Guam (Fig. 1), suggesting that gamete incompatibilities are a common feature of these urchins in spite of their close relationship. We have examined the steps of fertilization in crosses between Hawaiian *Echinometra* species in order to determine which ones are species-specific.

Step 1: Sperm activation. For successful fertilization to occur, sperm must first undergo a complex physiological activation induced by components of egg jelly—this allows them to find the egg and penetrate the jelly (reviewed in Ward and Kopf, 1993). Specificity of these events is generally moderate; only distantly related urchins are incompatible. For example, the egg-jelly peptide speract can activate sperm from different genera of urchins (Trimmer and Vacquier, 1986; Garbers, 1989; Ward and Kopf, 1993). In crosses of *Strongylocentrotus,* these events are also not species-specific (Minor *et al.*, 1991). Likewise, in crosses of Hawaiian *Echinometra,* large numbers of het-

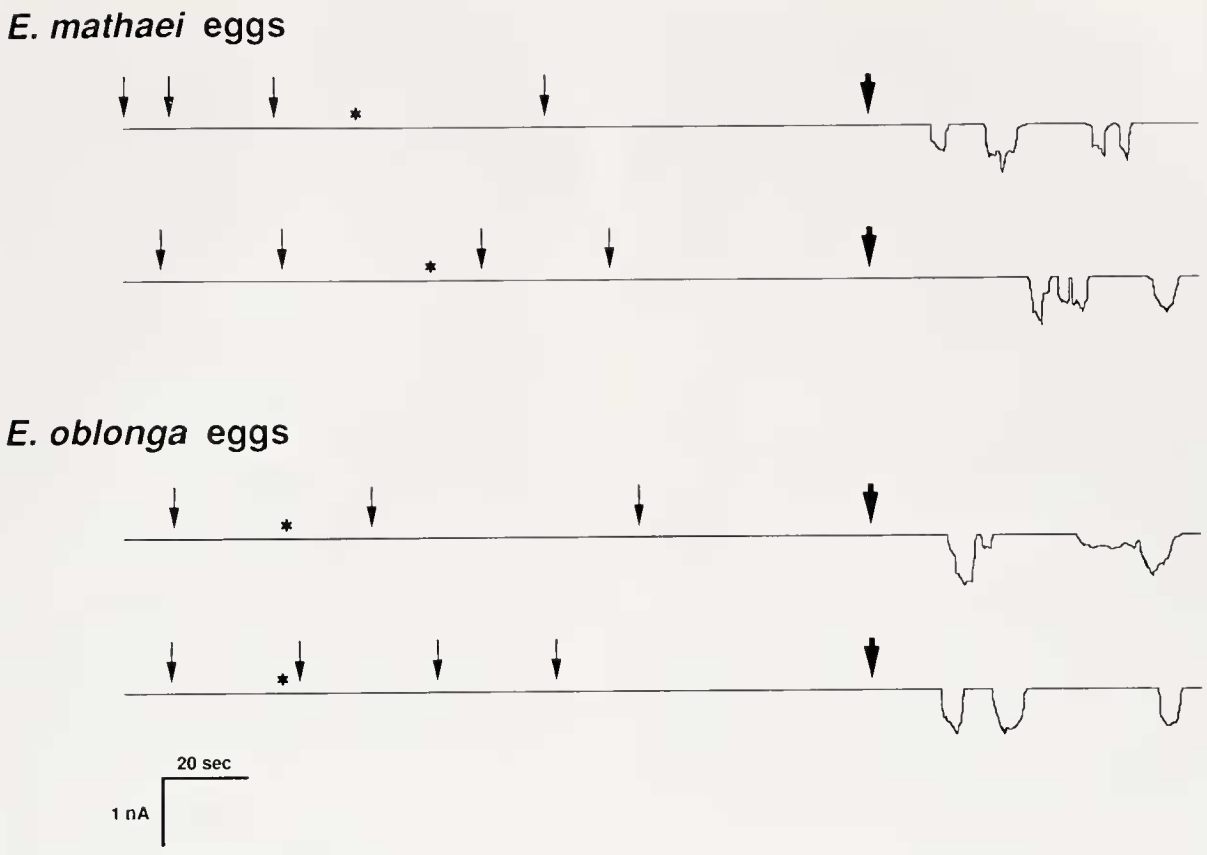

Figure 4. Portions of recordings of transmembrane current of *Echinometra* eggs voltage-clamped at approximately −75 mV. Small arrows indicate times of addition of aliquots of heterospecific sperm. Large arrow indicates time of addition of an equal aliquot of conspecific sperm. For clarity, the recordings have been aligned at the time of addition of conspecific sperm; the times of heterospecific gamete interaction are somewhat longer than is shown. The asterisk indicates the time at which all control eggs had elevated fertilization envelopes. Transient currents of approximately −0.5 nA occurring after addition of conspecific sperm indicate temporary electrical continuity between egg and sperm. In each of these trials, the voltage clamp was released, after which the impaled egg underwent normal fertilization depolarization and elevation of the fertilization envelope.

erospecific sperm rapidly arrive at the egg plasma membrane, suggesting that activation is readily achieved.

Step 2: Acrosome reaction. Although the acrosome reaction step is blocked in some crosses between divergent urchins (Segall and Lennarz, 1981), this step is typically not strongly and reciprocally species-specific. The acrosome reaction is not blocked in crosses between *Strongylocentrotus purpuratus* and *S. franciscanus* (reviewed in Minor *et al.*, 1989, 1991). In addition, the acrosome reaction has been shown to occur normally in most of the possible crosses between urchins from four different genera of Caribbean urchins, including *Echinometra lucunter*, and was not reciprocally blocked between any pair of these species (Summers and Hylander, 1975).

In Hawaiian *Echinometra* crosses, asymmetry was observed in the acrosome reaction step; in all crosses, however, more than half of the sperm approaching the egg surface underwent the acrosome reaction within 20 s. This degree of reaction is too high to account for the observation that only a small percentage of the eggs in both heterospecific crosses typically elevate fertilization envelopes. Even though induction of the acrosome reaction is measurably different depending on the direction of the cross, this step is not an effective barrier to cross-fertilization. The gamete interaction steps primarily responsible for preventing interspecific fertilization in both directions occur after the induction of the acrosome reaction.

Step 3: Sperm-egg attachment. In contrast to the preceding steps of gamete interaction, the attachment of sperm to the vitelline layer of eggs is species-specific in many urchin crosses (Summers and Hylander, 1975, 1976; Minor *et al.*, 1991). We found a similar pattern in crosses of the closely related Hawaiian *Echinometra* species, in which eggs bound severalfold fewer heterospecific sperm

than conspecific sperm (Fig. 2). These results agree with our previous counts of sperm attachment to dejellied eggs (Palumbi and Metz, 1991). It might be expected that heterospecific sperm would fertilize one-third to one-fifth of the eggs fertilizable by conspecific sperm, because the results show that eggs will bind approximately one-third to one-fifth as many heterospecific sperm as conspecific sperm. Typically, however, only a much smaller proportion of eggs in heterologous crosses eventually become fertilized. This indicates that sperm-egg attachment is not the only barrier to cross-fertilization in *Echinometra*. Under certain conditions, sperm from *Strongylocentrotus purpuratus* will attach to the vitelline layer of eggs from the highly divergent urchin *Arbacia punctulata*, yet only a small proportion of the eggs fertilize (Glabe *et al.*, 1981). On the basis of these observations, Glabe *et al.* suggested that in heterospecific crosses, bound sperm may not always be capable of penetrating the vitelline layer and developing continuity with the egg plasma membrane.

Step 4: Interactions of attached gametes and sperm-egg fusion. The best-known indicator of sperm-egg fusion is the fertilization envelope. However, elevation of the fertilization envelope is a complex process that normally occurs only after the egg has undergone a series of changes induced by fusion with the sperm (reviewed in Jaffe, 1986; Kay and Shapiro, 1985); hence it is an indicator of *completed* gamete fusion. By contrast, the earliest stages of the gamete fusion process are indicated by transmembrane electrical changes during interaction of the attached sperm and egg (Chambers, 1989; Whitaker *et al.*, 1989; Chambers and McCulloh, 1990). It is not yet known whether complete fusion of the sperm and egg plasma membranes is required for the sperm to influence the egg transmembrane potential. It is clear, however, that if plasma membranes have fused, then electrical continuity must exist between the gametes. Eggs held at the resting potential by voltage clamping are prevented from undergoing the normal depolarization that accompanies gamete fusion; instead, transient fusion of a sperm creates a transient transmembrane current (Chambers, 1989). We measured the incidence of these transient currents in voltage-clamped eggs to directly compare the ability of conspecific and heterospecific sperm to initiate the process of fusion. In reciprocal heterospecific crosses of Hawaiian *Echinometra* species, sperm were typically incapable of developing temporary electrical continuity with the egg. Thus species-specificity resides in the interactions of the sperm acrosomal surface and the egg vitelline layer that bring the plasma membranes together and allow fusion to occur.

Levels of reproductive isolation

Species-specific gamete interactions contribute directly to reproductive isolation between sea urchin species. In some cases, however, sympatric congeneric sea urchins fertilize readily in one or both directions of hybrid crosses (Shearer *et al.*, 1913; Strathmann, 1981; Lessios and Cunningham, 1990; Uehara *et al.*, 1990). When barriers are not present at fertilization, other types of reproductive isolation—including habitat segregation, timing of spawning, and postmating incompatibilities—are likely to be involved in maintaining distinctions between marine species (see review by Knowlton, 1993).

Hybrid sterility and inviability are important aspects of postmating reproductive isolation in *Drosophila* and other organisms (reviewed by Coyne and Orr, 1989; Coyne, 1992). However, because of difficulties rearing sea urchins, little is known about postmating isolating mechanisms in this group. In Hawaii, the existence of rare morphologically intermediate urchins suggests that viable and fertile hybrids may result from interspecific crosses between *E. mathaei* and *E. oblonga* in nature (Kelso, 1970; Palumbi and Metz, 1991). Although the strength of postmating isolation remains unclear, the strong gamete incompatibilities that have evolved act first during reproduction and are probably most important in maintaining barriers to gene flow in present populations.

Mechanisms of gamete incompatibility

In the genus *Strongylocentrotus*, the sperm acrosomal protein bindin has been shown to species-specifically attach the sperm to a receptor on the egg vitelline layer (Vacquier and Moy, 1977; Glabe and Vacquier, 1977; Minor *et al.*, 1989, 1991; Lopez *et al.*, 1993; Foltz *et al.*, 1993; Foltz and Lennarz, 1993). In addition to its responsibility for attaching gametes, the bindin-receptor interaction is also likely to have a role in setting up plasma membrane fusion (Glabe, 1985; Foltz and Lennarz, 1993). The steps of fertilization that involve bindin correspond to the steps that are reciprocally species-specific in closely related *Echinometra* congeners.

Bindin and its receptor could contribute in two ways to the reciprocal barrier to fertilization between Hawaiian *Echinometra* species. First, heterospecific bindin might not be properly recognized by the receptor on the egg vitelline layer, resulting in the reduced gamete attachment that we observe. Second, mismatched heterologous bindin-receptor complexes that occasionally succeed in attaching gametes might not be capable of undergoing subsequent molecular interactions associated with membrane fusion. This could result in the barrier to the formation of continuity of the sperm and egg plasma membranes that we observe. Other recognition molecules are probably also involved in the barrier to interspecific fertilization, especially during the complex process of gamete fusion (Whitaker *et al.*, 1989; Foltz and Lennarz, 1993).

Which fertilization steps are evolutionarily labile?

Species-specific fertilization arises as gamete recognition molecules differentiate, so recognition molecules that have the most freedom to vary within populations over time are likely to be the ones that first develop species-specificity. In sea urchins, different steps of fertilization are not equally likely to become blocked between species. The responses of the sperm to egg jelly, including the acrosome reaction, are often not strongly and reciprocally species-specific, except in crosses between divergent species (Summers and Hylander, 1975; Minor *et al.*, 1991). Components of egg jelly, including a fucose sulfate glycoconjugate and sperm-activating peptides, operate together to initiate the acrosome reaction (reviewed in Ward and Kopf, 1993). In addition, these molecules initiate ionic movements across the sperm membrane and cAMP-dependent protein kinase activity in sperm associated with sperm activation (reviewed in Trimmer and Vacquier, 1986; Ward and Kopf, 1993). Multiple functions (and in the case of the fucose sulfate glycoconjugate, a complex biosynthetic pathway), are likely to create constraints on the variability of the egg jelly components. Constraints such as these could prevent rapid differentiation of species-specificity.

The attachment and fusion steps of sea urchin fertilization, which are most often species-specific, are likely to be mediated by molecules that have more freedom to differentiate. Reciprocally incompatible attachment and fusion suggest that two complementary recognition molecules, such as bindin and its receptor, both differentiate between species. In *Strongylocentrotus* bindin and its receptor, protein-protein recognition involving direct binding of polypeptides expressed on the two gametes has been found. Unmodified bindin polypeptide attaches to the egg surface (Lopez *et al.*, 1993), and conversely, a region of the receptor polypeptide binds to bindin particles (Foltz *et al.*, 1993). In addition, treatment of eggs with a peptide derived from bindin can inhibit fertilization, presumably by binding to the receptor (Minor *et al.*, 1993). All of these effects are species-specific and support the view that recognition and binding result primarily from interactions of polypeptide domains. As species differentiate, genetic changes could act directly on the amino acid sequence of recognition domains in proteins expressed on sperm and eggs. Changes in the surface proteins of both the male and the female gametes would likely be required for differentiation of recognition. Constraints on such changes may be substantial, but would be lessened if they were uncoupled from other functional constraints of cell physiology. Accordingly, recognition domains of proteins might be susceptible to relatively rapid differentiation, and gamete interaction steps mediated by this type of recognition might reasonably be the first to evolve reciprocal species-specificity and premating reproductive isolation.

Reciprocal gamete incompatibility between recently diverged *Echinometra* species occurs during the stages of fertilization in which bindin and its receptor play a major role. We recently obtained the nucleotide sequences of bindin genes from these urchins and found that they are substantially differentiated (E. C. Metz and S. R. Palumbi, in preparation). Further studies of the Indo-Pacific *Echinometra* species group may aid our understanding of how gamete recognition evolves and contributes to differentiation of species.

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